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The Interaction between Polysaccharides and other Macromolecules

9. THE EXCLUSION OF MOLECULES FROM HYALURONIC ACID GELS AND SOLUTIONS*

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The extent to which hyaluronic acid can exclude other macromolecules from its molecular domain has long been a matter of controversy. Johnston (1955) calculated an impenetrable volume for albumin of 10^3 ml./g. of polysaccharide from sedimentation experiments with albumin and hyaluronic acid, but his results may be due to effects other than an exclusion (Laurent & Pietruszkiewicz, 1961). Blumberg & Ogston (1956) and Ogston & Phelps (1961) studied the sedimentation of hyaluronic acid in the presence of various concentrations of serum albumin and β_A-lactoglobulin. Their experiments indicated that only 12 ml. of solvent/g. of polysaccharide could be considered inaccessible to the proteins. However, simultaneous experiments with equilibrium dialysis

* Part 8: Laurent & Killander (1964).

(Ogston & Phelps, 1961) indicated a much larger excluded volume of about 300 ml./g. for the same proteins. From the measurements of the osmotic pressure of mixtures of hyaluronic acid and albumin, Laurent & Ogston (1963) calculated an excluded volume of approx. 25 ml./g. at low polysaccharide concentrations.

Because the interactions between polysaccharides and other macromolecules might be important physiologically, it is desirable to resolve the discrepancies mentioned above. To this end, the author has examined the exclusion of various substances from solutions and gels of hyaluronic acid. Two methods have been used: namely, chromatography on a bed of gel grains (gel filtration) and equilibrium dialysis. The exclusion due only to the steric configuration of the polysaccharide was determined by choosing systems in which electrostatic interactions and the formation of complexes between the polysaccharide and the other substances were supposedly negligible.

EXPERIMENTAL

Materials

Hyaluronic acid. The polysaccharide was prepared from human umbilical cords and had the same properties as the batches used in previous investigations (Laurent & Pietruszkiewicz, 1961; Laurent & Ogston, 1963). Stock solutions of hyaluronic acid (approx. 0·01 g./ml.) were prepared in 0·2 M-sodium chloride. Dilutions of these were made by weighing appropriate amounts.

Hyaluronic acid gel. The gel was prepared from 1·35 g. of ethanol-precipitated hyaluronic acid as described by Laurent, Hellsing & Gelotte (1964). The concentration of hyaluronic acid was 0·12 g./ml. during the cross-linking procedure. The gel was first allowed to swell in distilled water for 2 days before equilibration with 0·1 M-sodium chloride–0·05 M-tris buffer, pH 8·5. The swollen gel was disintegrated by a short run in a Waring Blendor and fine particles were removed by sieving through a 0·25 mm.-mesh screen.

Proteins. The equine cyanmethaemoglobin was that used by Laurent (1963*b*). Human serum albumin (lot no. Rd 023), human 7 S γ-globulin (lot no. 65549), human transferrin (lot no. Rfe 58) and human caeruloplasmin (lot no. DwZ 144) were kindly supplied by AB Kabi, Stockholm. The albumin and γ-globulin preparations were the same preparations as those used in previous investigations (Laurent, Björk, Pietruszkiewicz & Persson, 1963; Laurent & Ogston, 1963). Equine myoglobin was kindly supplied by Dr A. Ehrenberg, Stockholm. Bovine α-crystallin was prepared electrophoretically as described by Björk (1960). Similar preparations were used in previous investigations (Laurent *et al.* 1963).

Sugars. Analytical-grade commercial preparations of glucose, sucrose and raffinose were used. Dextran was obtained from AB Pharmacia, Uppsala. Two preparations, dextran-500 (lot no. To 4935) and dextran-2000 (lot no. FDR 922) with weight-average molecular weights of $4{\cdot}5 \times 10^5$ and 2×10^6, were used.

Methods

Gel filtration. The fraction of the hyaluronic acid gel containing particles greater than 0·25 mm. in diameter was packed to a depth of 10·6 cm. into a column with a diameter of 2·6 cm. under a hydrostatic head of 60 cm. The total volume of the bed was 57 ml. A filter paper was placed on the top of the column. The column was then equilibrated with 1–2 bed volumes of 0·1 M-sodium chloride–0·05 M-tris buffer, pH 8·5, containing 25% (v/v) of a saturated solution of 5,7-dichloro-8-quinolinol (a chelating agent), before use. This medium was used as eluent in all experiments except the last, where it was replaced by 0·1 M-sodium chloride–0·05 M-glycine buffer, pH 9·5. The change in buffer did not affect the size or void volume of the gel bed.

The chromatography was performed at 4°. The column was operated at 1·5 ml./hr. under a hydrostatic head of 60 cm. Samples (1 ml.) were applied at the top of the column by layering the denser sample solution below a layer of buffer. Approx. 1 ml. fractions were collected and their volumes were determined accurately by weighing the collector tubes before and after the experiments. The tubes were closed with rubber stoppers after collection to prevent evaporation. The elution volumes of the various substances were measured from the point at which half the sample had entered the column. Correction was made for the volume of the fluid trapped in the outlet of the chromatographic tube.

Chromatographic runs were performed with up to three different substances, chosen such that they could be determined independently of each other. The following substances were used in the amounts indicated: myoglobin, 14 mg.; cyanmethaemoglobin, 20 mg.; α-crystallin, 20 mg.; albumin, 40 mg.; γ-globulin, 40 mg.; transferrin, 40 mg.; caeruloplasmin, 40 mg.; the dextrans, 10 or 20 mg.; glucose, 20 mg.; sucrose, 20 mg.; raffinose, 20 mg.

Equilibrium dialysis. The experiments were performed at 4° in cells with the dimensions of those used by Ogston & Phelps (1961). The two compartments, one containing hyaluronic acid and the other buffer, were separated by a Millipore HA membrane (Millipore Filter Corp., Watertown, Mass., U.S.A.) with pores 0·45 μ in diameter. The membrane was completely impermeable to hyaluronic acid, since no material containing uronic acid was found in the buffer compartment at the end of the dialysis experiments. The membrane was permeable to all proteins tested.

The experiments were carried out essentially as described by Ogston & Phelps (1961). Reciprocal experiments, one in which the protein was present initially in the hyaluronic acid compartment and one in which the protein was introduced into the buffer compartment, were performed at each hyaluronic acid concentration used: the equilibrium was approached from both sides. The cells were rotated during the experiments. Usually 130–170 hr. was required for equilibration. Samples for protein analyses were taken from each compartment at 12 hr. intervals. The hyaluronic acid concentration was checked at the end of all runs except those performed with cyanmethaemoglobin.

Series of experiments were carried out with four proteins, i.e. cyanmethaemoglobin, serum albumin, γ-globulin and α-crystallin. Experiments with albumin were performed in 0·2 M-sodium chloride–0·05 M-phosphate buffer, pH 7·2, and experiments with γ-globulin were made in 0·2 M-sodium chloride–0·1 M-glycine buffer, pH 9·0. The average concen-

tration of protein in the two compartments was 2×10^{-2} g./ml. in all experiments with these proteins.

Experiments with cyanmethaemoglobin were carried out in 0·1 M-sodium chloride–0·05 M-tris buffer, pH 9·0. The average concentration of protein was 10^{-2} g./ml. Experiments with α-crystallin were carried out in 0·2 M-sodium chloride–0·05 M-phosphate buffer, pH 7·6, containing 10% (v/v) of saturated 5,7-dichloro-8-quinolinol. The average concentration of protein was $2·5 \times 10^{-3}$ g./ml.

Chemical analyses. Hyaluronic acid was determined by the carbazole method (Dische, 1947; Laurent, Ryan & Pietruszkiewicz, 1960). The concentration of organic matter in the hyaluronic acid gel was determined as the difference between dry weight and ash value. The gel was dried to constant weight at 98° at a pressure of 0·02 mm. Hg over phosphorus pentoxide. Before the determination, the gel was washed repeatedly with 0·15 M-sodium chloride to remove tris buffer and then was packed tightly by centrifugation at 9000 rev./min. in an ordinary laboratory centrifuge to remove interstitial water between the gel grains.

The fractions obtained in the gel-filtration experiments were analysed after appropriate dilutions: cyanmethaemoglobin and myoglobin were determined spectrophotometrically at 420 mμ; albumin, γ-globulin, transferrin, caeruloplasmin and α-crystallin were determined spectrophotometrically at 280 mμ; sugars were determined by a modification of the anthrone reaction (Dreywood, 1946).

In the dialysis experiments protein concentrations were determined by the biuret reaction (Dittebrandt, 1948). The background colour due to hyaluronic acid represented less than 2% of the total extinction in all experiments. Protein concentrations were calculated from the net extinction after subtraction of this small blank. The concentration of cyanmethaemoglobin was determined directly by extinction measurements at 540 mμ.

RESULTS

Gel filtration. The hyaluronic acid gel used contained $1·45 \times 10^{-2}$ g. of hyaluronic acid/ml. Some typical chromatograms are shown in Figs. 1 (*a*)–(*e*). Chromatograms obtained with γ-globulin at pH 8·5 and 9·5 were nearly identical. γ-Globulin, serum albumin and transferrin all contained some materials that were eluted ahead of the respective main components. With γ-globulin and serum albumin the faster-moving components are probably aggregates or polymers of the proteins, but the presence of trace impurities cannot be ruled out.

Dextran-2000 is assumed to be completely excluded from the gel and the void volume calculated from its elution peak is 12·8 ml., or 22·4% of the total bed volume. $K_{av.}$, the fraction of the gel available to a substance, was defined by Laurent & Killander (1964) as:

$$K_{av.} = \frac{V_e - V_0}{V_t - V_0} \qquad (1)$$

where V_e is the elution volume, V_0 the void volume and V_t the total bed volume. The $K_{av.}$ values calculated for the various substances are given in Table 1. For most substances, the elution volume is taken as the effluent volume at which the solute has its maximum concentration. However, with α-crystallin, which emerged in a rather asymmetrical zone, the elution volume was taken as the effluent volume at which half the solute had been eluted (H. Bennich, J. Killander & T. C. Laurent, unpublished work). With γ-globulin, serum albumin and transferrin, calculations were made for the main components.

Equilibrium dialysis. The equilibration followed closely the time-course described by Ogston & Phelps (1961). The partition of the proteins between the hyaluronic acid compartment and the buffer compartment at equilibrium was a function of the hyaluronic acid concentration. The results are plotted in Fig. 2.

DISCUSSION

Laurent & Killander (1964) showed that the separation of substances by chromatography on dextran gels could be attributed to differential exclusion resulting from steric interaction between the substances and a three-dimensional network of straight dextran chains. These chains should have a diameter of 14×10^{-8} cm. and a total length about 4–5-fold shorter than the combined length of all monosaccharides in the gels. The treatment of Laurent & Killander gave a reasonable explanation of the gel-filtration process and provided some valuable information about the structure of the dextran gel on a macromolecular level. A comparison of gel-filtration results with the results of studies on the solubility of proteins in dextran solutions (Laurent, 1963*a*, *b*) indicated that the exclusion properties of dextran gels and dextran solutions were analogous.

The successful correlation of gel-filtration results with the exclusion properties of dextran solutions suggested similar studies with hyaluronic acid. Since cross-linked hyaluronic acid gels can be prepared without any detectable destruction of the amino and carboxyl groups on the chain (Laurent *et al.* 1964), such experiments should be valid.

To determine the exclusion due to steric interactions alone, electrostatic interactions were suppressed by performing the chromatography at high ionic strength and at high pH. Some experiments (T. C. Laurent, unpublished work) indicated that electrostatic interactions occur when a protein is chromatographed at pH values close to its isoelectric point, with the result that steric-exclusion effects are obscured by ion-exchange chromatography. That this is not the case in the present experiments is shown by the calculations below. The exclusion values are those that are optimum for a polysaccharide, and an adsorption of proteins

to the column material should have resulted in too low values of the calculated excluded volumes.

The values of $K_{av.}$ recorded in Table 1 have been plotted against the radii of equivalent spheres with the same diffusion constants as the respective substances (Fig. 3). Similar plots were constructed in the studies with dextran gels (Laurent & Killander, 1964). The radii of equivalent spheres listed in Table 1 were taken from previous investigations (Laurent & Killander, 1964; Laurent *et al.* 1963).

According to Ogston (1958), the volume fraction available to a sphere in a network composed of randomly oriented straight rods of infinite length should be

$$K_{av.} = \exp|-\pi L(r_s+r_r)^2| \quad (2)$$

where L is the concentration of rods expressed in cm./cm.^{3}, r_s is the radius of the sphere, and r_r is the radius of the rod. The continuous line in Fig. 3 has been drawn according to eqn. 2. L was calculated from the concentration of the gel, $1{\cdot}45 \times 10^{-2}$ g./

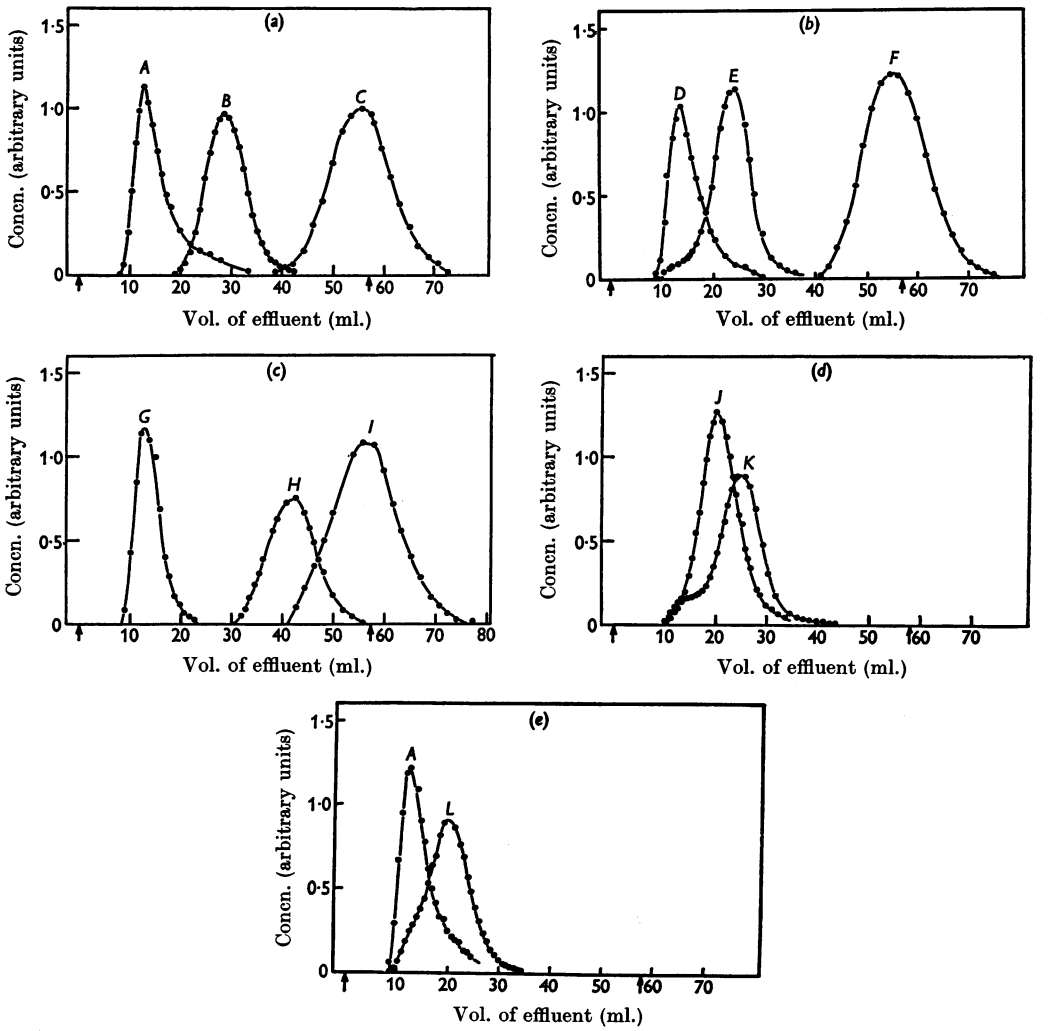

Fig. 1. Chromatograms of various substances on the hyaluronic acid gel: (*a*) dextran-2000 (*A*), cyanmethaemoglobin (*B*), sucrose (*C*); (*b*) dextran-500 (*D*), albumin (*E*), raffinose (*F*); (*c*) α-crystallin (*G*), myoglobin (*H*), glucose (*I*); (*d*) caeruloplasmin (*J*), transferrin (*K*); (*e*) dextran-2000 (*A*) and γ-globulin (*L*). The two arrows indicate respectively the start of the chromatograms and the total volume of the gel bed. Caeruloplasmin (*J*) and transferrin (*K*) (Fig. 1*d*) were chromatographed separately, but their elution curves have been drawn in the same graph. All chromatograms were made at pH 8·5 except that with γ-globulin (*L*) (Fig. 1*e*), which was made at pH 9·5. Experimental details are given in the text.

ml., by assuming that the gel contained only hyaluronic acid and that the latter substance is a straight chain of disaccharides with a disaccharide weight of 379 ($C_{14}H_{21}NO_{11}$) and a disaccharide length of $10{\cdot}3 \times 10^{-8}$ cm. r_r was estimated to be $3{\cdot}5 \times 10^{-8}$ cm.

The good agreement between the calculated curve and the experimental points in Fig. 3 supports the hypothesis that the exclusion is attributable to the steric properties of a network of linear polysaccharide chains. In its ability to cause exclusion of other substances, hyaluronic acid is 4–5 times as effective as dextran, which is branched and less rigid.

Table 1. *Results of gel-filtration experiments with various substances on hyaluronic acid gel*

Experimental details are given in the text. The radii of equivalent spheres are from Laurent & Killander (1964) and Laurent *et al.* (1963).

Substance	$K_{av.}$	$10^8 \times$ Radius of equivalent sphere (cm.)
Glucose	0·99	3·9
Sucrose	0·96	5·1
Raffinose	0·93	6·2
Myoglobin	0·66	18·8; 20·7*
Cyanmethaemoglobin	0·36	31·3†
Serum albumin	0·25	34·9; 36·1*
Transferrin	0·27	36·1; 40·2*
Caeruloplasmin	0·17	45·3; 47·3; 56·1*
7 s γ-Globulin	0·16	55·5
α-Crystallin	0·014	97

* Several values have been published for the diffusion constant of the same protein.

† From the diffusion constant of haemoglobin.

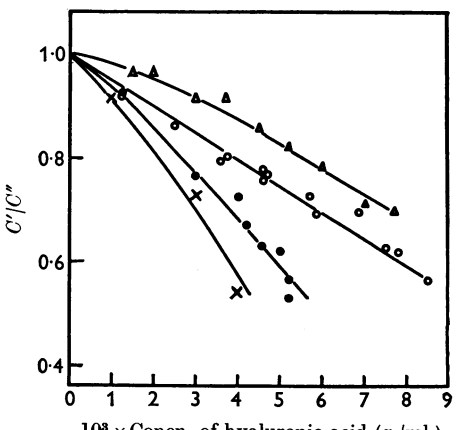

Fig. 2. Results of the equilibrium-dialysis experiments. The ratio of the protein concentration in the hyaluronic acid phase, C', to that in the buffer phase, C'', was plotted against hyaluronic acid concentration. △, Cyanmethaemoglobin; ○, serum albumin; ●, γ-globulin; ×, α-crystallin. Experimental details are given in the text.

Two of the points in Fig. 3 (γ-globulin and caeruloplasmin) fall rather far from the theoretical curve. These substances also deviate from theory when chromatographed on dextran gels. In both instances the deviations might be at least partly due to uncertainties in calculating the radius of the equivalent spheres. Most of the published diffusion data for 7 s γ-globulins were probably obtained with preparations that contained some higher-molecular-weight material. Also, there are large discrepancies between the various values reported in the literature for the diffusion constant of caeruloplasmin.

The previous study showed that, for some proteins, solutions containing 5 and 10 % of dextran provided the same exclusion as dextran gels of the same respective concentrations. Analogous studies have not been carried out with hyaluronic acid, since it is not possible to perform experiments with solutions containing $1{\cdot}45 \times 10^{-2}$ g. of hyaluronic acid/ml. However, if one assumes that solutions of hyaluronic acid retain the exclusion properties of the gel, extrapolation of the gel-filtration results provides a value of about 80 ml./g. of polysaccharide for the exclusion of serum albumin at low hyaluronic acid concentrations. This is considerably lower than the value obtained by Ogston & Phelps (1961) and higher than that given by Blumberg & Ogston (1956) and by Laurent & Ogston (1963). Further, Ogston & Phelps were unable to correlate their results with eqn. (2).

In an attempt to resolve these discrepancies, equilibrium dialysis was performed in four series of experiments where cyanmethaemoglobin, serum

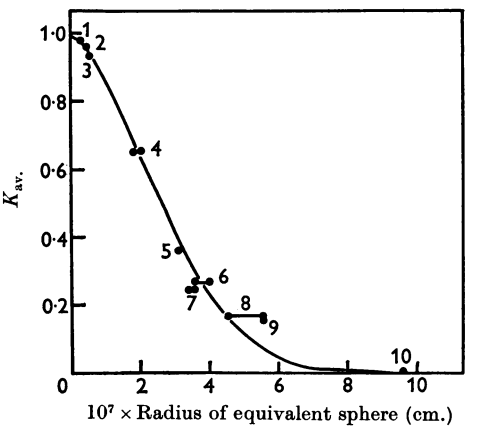

Fig. 3. Comparison of experimental gel-filtration results with theory. The points represent: glucose (1); sucrose (2); raffinose (3); myoglobin (4); cyanmethaemoglobin (5); transferrin (6); serum albumin (7); caeruloplasmin (8); γ-globulin (9); α-crystallin (10). Experimental details are given in the text. Points connected by horizontal lines indicate the spread of values given in the literature for the radius of the equivalent sphere for the same protein.

albumin, γ-globulin and α-crystallin were partitioned between a hyaluronic acid phase and a buffer phase. The results (Fig. 2) were different from those reported by Ogston & Phelps (1961). The partition coefficients reported by the latter authors for serum albumin and hemoglobin derivatives in 0·09 % hyaluronic acid were obtained in the present experiments only when the concentration of polysaccharide was increased to ten times that value.

Fig. 1 shows that both the albumin and the γ-globulin contain some high-molecular-weight contaminants that might tend to lower the C'/C'' values in Fig. 2, but probably not significantly.

If one assumes that the relative effect of hyaluronic acid on the activity of the protein is essentially independent of the protein concentration in the experimental region, as the findings of Ogston & Phelps (1961) and Laurent & Ogston (1963) indicate, then the present partition data can be regarded as a measure of the volume available to the substance in the presence of the polysaccharide and can be compared with the gel-filtration results. According to eqn. (2), $-\log K_{av.}$ should be proportional to L, i.e. to the concentration of hyaluronic acid. This has been tested in Fig. 4, where the results from gel filtration and equilibrium dialysis have been plotted simultaneously. All the lines show an upward curvature, indicating that the exclusion properties of hyaluronic acid at low concentrations are not of the magnitude expected for a rigid three-dimensional network. Apparently the network model does not hold at low concentrations where the entanglement of the polysaccharide chains decreases. Although the concentration of polymer chains is probably fairly uniform throughout the solution at relatively high polymer concentrations, the macromolecules will separate from each other, giving rise to considerable concentration fluctuations in dilute solutions.

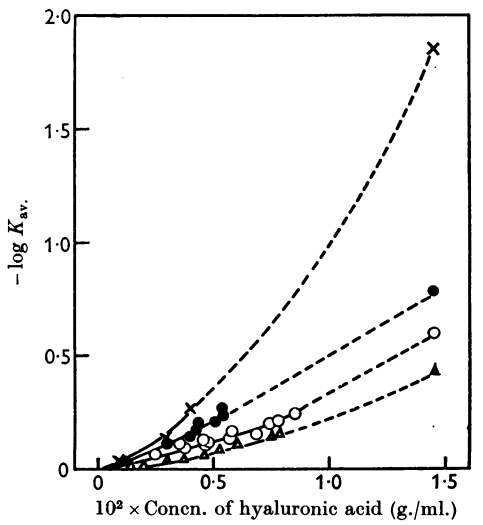

Fig. 4. Comparison of the excluded volumes obtained in hyaluronic acid solutions (points up to $1·0 \times 10^{-2}$ g./ml.) with those determined with the hyaluronic acid gel (points at $1·45 \times 10^{-2}$ g./ml.). △, Cyanmethaemoglobin; ○, serum albumin; ●, γ-globulin; ×, α-crystallin. Experimental details are given in the text.

The partition coefficients for serum albumin are in good agreement with the osmometric results obtained by Laurent & Ogston (1963). Fig. 2 yields C'/C'' values of 0·77 and 0·59 for albumin at hyaluronic acid concentrations of $4·4 \times 10^{-3}$ and 8×10^{-3} g./ml. respectively. The osmotic measurements at these concentrations predicted values of 0·78 and 0·56.

The work of Laurent & Killander (1964) and the present work indicate that gel filtration may be a very useful method for investigating the structures of other gels, e.g. those of agar, fibrin and collagen. Such structural information is essential to an understanding of the polymerization process by which these gels are formed. Some results from gel filtration on agar gels have been published by Andrews (1962). It is not possible to draw any definite conclusions from his results, except that the polymer chains in the agar gel must be considerably thicker than those in dextran and hyaluronic acid gels if agar is to be treated as a three-dimensional network of rigid rods.

SUMMARY

1. A number of substances have been chromatographed on a cross-linked hyaluronic acid gel with a concentration of $1·45 \times 10^{-2}$ g./ml. at high ionic strength and at high pH.

2. The results are interpreted on the basis of a steric exclusion of the substances from the gel. Calculations show that the results are compatible with the hypothesis that hyaluronic acid forms a continuous network of extended rigid linear polysaccharide chains in the gel.

3. The excluded volumes obtained by equilibrium dialysis of proteins between a hyaluronic acid phase and a buffer phase at polysaccharide concentrations of 1×10^{-3}–8×10^{-3} g./ml. were somewhat lower than those obtained in the gel experiments, indicating that the network model breaks down at lower concentrations. The results are in good agreement with previous osmotic results.

4. Gel filtration should be a useful tool for the structural analysis of gels.

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Carbohydrate Synthesis from Lactate in Pigeon-Liver Homogenate

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Liver has long been known to be a major site of carbohydrate formation from lactate (Cori & Cori, 1929), but in the past it has not proved possible to reproduce the synthesis satisfactorily in isolated tissue *in vitro*. It is true that liver slices suspended in a saline medium synthesize some carbohydrate (Takane, 1926; Buchanan, Hastings & Nesbett, 1942, 1949), but for reasons that are still obscure the rate of carbohydrate synthesis in slices is very much lower than the rate *in vivo*. There are no records of experiments in which the synthesis occurred in disrupted liver cells. Since cell-free material is a prerequisite for the detailed investigation of the process, efforts were made to obtain a cell-free liver preparation capable of synthesizing carbohydrate at high rates. A pigeon-liver homogenate is described below which readily forms carbohydrate from L-lactate.

EXPERIMENTAL

Analytical methods. Glucose and the sum of glycogen and glucose were determined enzymically by the glucose-oxidase method (see Krebs, Bennett, de Gasquet, Gascoyne & Yoshida, 1963). 'Bound' glycogen, i.e. glycogen insoluble in aqueous $HClO_4$, was determined by washing the centrifuged and well-drained sediment from the $HClO_4$ treatment twice with 2% (w/v) $HClO_4$ solution. The washed precipitate was dissolved in 1 ml. of 30% (w/v) KOH and heated in a boiling-water bath for 20 min. The glycogen was isolated according to the method of Good, Kramer & Somogyi (1933) and determined as described by Krebs *et al.* (1963).

When glucose is determined by the glucose-oxidase method in the presence of glycogen or maltose the values obtained by the method of Krebs *et al.* (1963) are too high, because glucose-oxidase preparations, even of high purity, are contaminated by amylase, maltase and other glucosidases (Dahlqvist, 1961). In previous work on kidney this interference by glycogen was negligible because the amounts of glycogen occurring in kidney are very low, but it is not negligible in the liver. The difficulty can be overcome by including in the reagent tris (Dahlqvist, 1961), which inhibits amylase and glucosidases (Larner & Gillespie, 1956; Dahlqvist, 1958). The previous procedure was therefore modified as follows: a 1 ml. sample, deproteinized with $HClO_4$ (if necessary diluted with 2% $HClO_4$), is incubated with 2·5 ml. of a solution containing 4 mg. of peroxidase, 12·5 mg. of glucose oxidase (Sigma), 0·5 ml. of 1% (w/v) *o*-dianisidine in 95% (v/v) ethanol, and phosphate–tris buffer, pH 7·3, to 100 ml. This buffer is prepared by dissolving 56·79 g. of Na_2HPO_4 (anhydrous), 15·6 g. of $NaH_2PO_4,2H_2O$, 12·1 g. of tris and 43 ml. of 2N-HCl in a final volume of 1 l.

During the present work it became clear that avian (and mammalian) liver contains not only glucose, glycogen and the phosphorylated hexoses of glycolysis but also significant quantities of oligosaccharides (see Sie & Fishman, 1958; Olavarria, 1960; Olavarria & Torres, 1962). The oligosaccharides would appear as glycogen in the method used. As the work was concerned with the formation of carbohydrate from non-carbohydrate precursors it was the main object to determine the sum of glucose, glycogen and the intermediate oligo- or poly-saccharides. A resolution of the carbohydrates formed into the various fractions is under investigation.

In several experiments the glucose-oxidase method was checked by the hexokinase–glucose 6-phosphate-dehydrogenase method (see Slein, 1963) and good agreement was found.